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Differential Activation and Trafficking of μ-Opioid Receptors in Brain Slices

Seksiri Arttamangkul, Nidia Quillinan, Malcolm J. Low, Mark von Zastrow, John Pintar, and John T. Williams

Vollum Institute (S.A., N.Q., J.T.W.) and Center for the Study of Weight Regulation, Department of Behavioral Neuroscience (M.J.L.), Oregon Health and Science University, Portland, Oregon; Department of Psychiatry and Cellular and Molecular Pharmacology, University of California, San Francisco, California (M.v.Z.); and Department of Neuroscience and Cell Biology, Robert Wood Johnson Medical School, Piscataway, New Jersey (J.P.)

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ABSTRACT

The activation of G protein-coupled receptors results in a cascade of events that include acute signaling, desensitization, and internalization, and it is thought that not all agonists affect each process to the same extent. The early steps in opioid receptor signaling, including desensitization, have been characterized electrophysiologically using brain slice preparations, whereas most previous studies of opioid receptor trafficking have been conducted in heterologous cell models. This study used transgenic mice that express an epitope-tagged (FLAG) μ-opioid receptor (FLAGMOR) targeted to catecholamine neurons by regulatory elements from the tyrosine hydroxylase gene. Brain slices from these mice were used to study tagged MOR receptors in neurons of the locus ceruleus. Activation of the FLAG-MOR with [Met^5]enkephalin (ME) produced a hyperpolarization that desensitized acutely to the same extent as native MOR in slices from wild-type mice. A series of opioid agonists were then used to study desensitization and receptor trafficking in brain slices, which was monitored with a monoclonal antibody against the FLAG epitope (M1) conjugated to Alexa 594. Three patterns of receptor trafficking and desensitization were observed: 1) ME, etorphine, and methadone resulted in both receptor desensitization and internalization; 2) morphine and oxymorphone caused significant desensitization without evidence for internalization; and 3) oxycodone was ineffective in both processes. These results show that two distinct forms of signaling were differentially engaged depending on the agonist used to activate the receptor, and they support the hypothesis that ligand-specific regulation of opioid receptors occurs in neurons maintained in brain slices from adult animals.

The activation of G protein-coupled receptors results in a cascade of processes and different agonists acting on a single receptor can result in varying signaling patterns (Urban et al., 2007). The μ-opioid receptor (MOR) is a $G_{i/o}$-linked receptor that activates potassium conductance, inhibits voltage-dependent calcium conductance, and inhibits adenylyl cyclase (Williams et al., 2001). In the continued presence of agonist, the MOR desensitizes by a mechanism that is thought to be similar to that originally described for the β-adrenergic receptor (Krupnick and Benovic, 1998; Lefkowitz et al., 1998). The first step in receptor desensitization is thought to be a phosphorylation of the receptor by a G protein-coupled receptor kinase followed by the binding to β-arrestin with high affinity. This process also transforms the receptor into a state where it can no longer couple to G proteins and is therefore physiologically inactive. Acute desensitization can take place within a few minutes and requires a saturating concentration of agonist (von Zastrow et al., 2003; Connor et al., 2004; Gainetdinov et al., 2004).

Like the β-adrenergic receptor, the MOR is trafficked into early endosomes followed by a multistep process that recycles receptor back to the plasma membrane (Finn and Whistler, 2001; Tanowitz and von Zastrow, 2003). This trafficking

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ABBREVIATIONS: MOR, μ-opioid receptor; FLAGMOR-Tg/+, FLAG epitope-tagged μ-opioid receptor hemizygous transgenic mice; FLAGMOR-Tg/+, MOR−/−, homozygous μ-opioid receptor knockout mice (MOR-KO) crossed with the transgenic mice; FLAGMOR-Tg/+, Arr−/−, homozygous β-arrestin2-KO mice crossed with the transgenic mice; kb, kilobase(s); TH, tyrosine hydroxylase; ACSF, artificial cerebrospinal fluid; LC, locus coeruleus; ME, [Met^5]enkephalin; MK-810, 5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine (dizocilpine maleate); GIRK, G protein-linked inwardly rectifying potassium channel; UK14304, 5-bromo-6-[2-imidazolin-2-ylamino]quinoxaline; β-CNA, β-chlornaltrexamine; ANOVA, analysis of variance.

pathway has been hypothesized to be a critical step in the recovery from desensitization where phosphatase activity is necessary for the reinsertion of receptors into the plasma membrane (Ferguson et al., 1998; Luttrell and Lefkowitz, 2002; von Zastrow et al., 2003; Marie et al., 2006). The trafficking of MOR has been characterized in a variety of expression systems, although the ability to follow the internalization of receptors in neurons under more physiological conditions in real time has been limited.

Receptor activation, desensitization, and internalization have different concentration dependence or require distinct agonist/receptor dwell times. To understand the complexity of signaling from a single receptor, it is necessary to study two or more processes simultaneously or at least under identical conditions with a variety of agonists. This study measured acute signal transduction (membrane hyperpolarization), acute desensitization, and receptor internalization, resulting from the activation of an epitope-tagged MOR in a brain slice preparation.

Materials and Methods

FLAGMOR-Transgenic Mice. The transgene was constructed by first ligating an 8.5-kb genomic fragment of the rat tyrosine hydroxylase (TH) gene from plasmid pTH9000 (Min et al., 1994) containing 5′ regulatory sequences, the basal promoter, and 26 base pairs from the 5′ untranslated region in exon 1 of TH to a 0.7-kb cassette containing a heterologous intron 2 and splice donor/acceptor sites from the rabbit β-globin gene. The 2.2-kb FLAG epitope-tagged MOR cDNA with a bovine growth hormone polyA sequence was obtained from excision of plasmid pcDNA3 SSF-MOR (Kim and von Zastrow, 2003) and ligated 3′ to the β-globin intron in a pBluescript SK+ plasmid vector. The FLAG epitope (DYKDDDA) is immediately preceded by a modified influenza hemagglutinin signal peptide sequence that facilitates translocation of the modified receptor into the endoplasmic reticulum and production of functional protein. Cotranslational cleavage of this sequence by signal peptidase is essential for subsequent binding of the M1 antibody to the free amino-terminal end of the FLAG epitope. An 11.4-kb SalI-NotI transgene fragment was purified from the final construct and used for pronuclear microinjection into zygotes of B6D2 F_2 mice by standard techniques. Two independent founders were identified by polymerase chain reaction genotyping of genomic DNA and backcrossed with C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) to obtain N_1 hemizygous progeny for initial characterization. The one strain with readily detectable levels of FLAGMOR by immunofluorescence in brain slices was further maintained by continued backcrossing with C57BL/6J mice. All data were collected from these hemizygous FLAGMOR-Tg/+ mice ranging from generations N_1 to N_5. FLAGMOR-Tg/+ mice were also crossed with MOR (Schuller et al., 1999) and β-arrestin2 knockout mice (Bohn et al., 2000) to generate mice that were hemizygous for the FLAGMOR transgene and homozygous for either the MOR-KO (FLAGMOR-Tg/+, MOR−/−) or the β-arrestin2-KO (FLAGMOR-Tg/+, Arr−/−). All animal experiments were conducted in accordance with the National Institutes of Health guidelines and with approval from the Institutional Animal Care and Use Committee of the Oregon Health and Science University (Portland, OR).

Fluorescent Immunohistochemistry. Fixed brains from FLAGMOR-Tg/+ mice perfused with 4% paraformaldehyde were prepared and sliced to 50 μm using a vibratome (Leica, Nussloch, Germany) as described previously (Ford et al., 2006). The primary antibody against the FLAG epitope (M1; Sigma-Aldrich, St. Louis, MO) was chemically linked to Alexa 555 (monoclonal labeling kit; Invitrogen, Carlsbad, CA) and used at a 1:500 dilution. Immunostaining was done using a free-floating method, and tissue slices were incubated at 4°C for 18 h. For tyrosine hydroxylase colocalization experiments, the tissues were permeabilized with 0.4% Triton X. The primary antibody against TH was a mouse monoclonal antibody (1:5000; IncStar, Stillwater, MN), and the secondary antibody was Alexa 488-labeled goat anti-mouse (1:1000; Invitrogen). Images were collected from a microscope equipped with a confocal system (Olympus, Center Valley, PA), excitation/emission at 488 and 543 nm for Alexa 488 and Alexa 555, respectively.

Electrophysiology. Adult mice (4–10 weeks) were used for electrophysiology experiments. Animals were anesthetized with isoflurane. Then, the brain was removed and sliced horizontally using a vibratome (Leica) in ice-cold artificial cerebrospinal fluid (ACSF) containing the following: 126 mM NaCl, 2.5 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 2.4 mM $CaCl_2$, 21.4 mM $NaHCO_3$, and 11 mM glucose. Slices (275 μm in thickness) were incubated in warm (34°C) oxygenated ACSF containing (+)-MK-801 (10 μM; Sigma-Aldrich) for at least 30 min before use in experiments. Sharp glass electrodes (50–60 MΩ) filled with 2 M KCl were used for intracellular recordings of membrane potentials. Experiments were performed at 35°C. Data were collected using Power Lab (Chart version 5.4; ADInstruments, Colorado Springs, CO) and acquired at 200 Hz. Drugs were applied by perfusion at the rate of 1.5 ml/min.

Two-Photon Microscopy. Brain slices (200–220 μm) were prepared similarly to those described for electrophysiological experiments. Slices were incubated in a solution containing M1 antibody (Sigma-Aldrich) conjugated with 10 μg/ml Alexa 594 (Invitrogen) for 45 to 60 min. In previous work, the behavior of antibody-tagged receptors with unlabeled receptors was compared, and there was no significant difference between the two conditions on the time scale of an hour (Whistler et al., 1999; Tanowitz and von Zastrow, 2003). The tissue was visualized with an upright microscope (Olympus) equipped with a custom-built two-photon apparatus. Data were acquired and collected using Scan Image software (Pologruto et al., 2003). A z-series was collected at 1-μm intervals for 15 μm. Drugs were applied by perfusion. All experiments were done at 35°C. Drugs used were [Met^5]enkephalin (ME), oxycodone (Sigma-Aldrich), oxymorphone (Mallinkrodt, Hazelwood, MO), and methadone, morphine, and etorphine (National Institute on Drug Abuse, Rockville, MD).

Quantification of Receptor Internalization. Analyses were done off-line with ImageJ software (National Institutes of Health, Bethesda, MD). Images were selected with the Stacks-Reducing Plugin algorithm for quantification. The stack was Z-projected using Sum-Slices method. Five random regions of interest were selected and averaged for background fluorescence. The average background fluorescence was then subtracted from the total fluorescent intensity of the whole frame. In control, data were obtained from slices before drug application. This fluorescent intensity was considered as total fluorescent receptors (C). After drug perfusion (15 min) followed by calcium-free ACSF containing 0.5 mM EGTA (10 min), a stack of images was collected and analyzed. This fluorescent intensity was termed internalized receptors (I). Percentage of internalization was calculated by (I/C) × 100, and averaged fluorescence from the drug-free controls was subtracted.

Radioligand Binding Assay. Slices containing the locus coeruleus (LC) nuclei were prepared (~500 μm), and the area of the LC was dissected. The tissue was homogenized using a Dounce tissue grinder in ice-cold 50 mM Tris-HCl, pH 7.4. Tissue from six to 10 animals was prepared for a binding assay using [^{3}H]diprenorphine (GE Healthcare, Chalfont St. Giles, UK) as described previously (Bunzow et al., 1995). Analyses were done using a one-site binding hyperbolic equation from Prism (GraphPad Software Inc., San Diego, CA).

Results

FLAGMOR-Transgenic Mice. A transgenic mouse strain was generated that expresses a fusion protein consisting of the MOR with an amino-terminal FLAG epitope targeted to cate-

cholamine neurons by an 8.5-kb segment of genomic DNA from the rat TH gene (Fig. 1A). Staining of FLAGMOR on the plasma membrane of LC neurons was demonstrated using the M1 antibody conjugated with Alexa 555 and was limited to the area of LC and colocalized with TH immunoreactivity (Fig. 1B). The FLAGMOR was also detected in other catecholamine neurons in the olfactory bulb, arcuate nucleus, substantia nigra, and ventral tegmental area (data not shown). There was no obvious phenotypic difference between the transgenic mice and their wild-type littermates. Hemizygous FLAGMOR-Tg/+ mice grew and bred normally.

Opioid Receptor Expression and Function. Receptor number determined by a binding assay using [^{3}H]diprenorphine indicated that LC neurons from the FLAGMOR-Tg/+ mice expressed approximately 2-fold more MOR than wild-type littermates (B_{max} = 732 ± 220 fmol/mg protein, n = 3 versus 364 ± 90 fmol/mg protein, n = 4). The K_d value from both groups of animals was the same (K_d = 0.36 ± 0.03 nM, n = 3 for FLAGMOR-Tg/+ and K_d = 0.46 ± 0.08 nM, n = 4 for wild type; t test, $P > 0.05$). This increased expression is similar to that reported recently for the δ-opioid receptor fused to enhanced green fluorescent protein (Scherrer et al., 2006). In homozygous δ-opioid receptor-enhanced green fluorescent protein knockin mice, there was about a 2-fold increase in binding sites and a 3-fold increase in guanosine 5′-*O*-(3-thio)triphosphate binding (Scherrer et al., 2006).

Functional coupling of opioid receptors to a G protein-linked inwardly rectifying potassium channel (GIRK) was measured by intracellular recording of membrane potential in LC neurons (Fig. 1C). The maximal hyperpolarization induced by 30 μM ME was 30.1 ± 1.3 and 28.8 ± 1.1 mV in FLAGMOR-Tg/+ and wild-type mice (n = 14 and 19 neurons, respectively; t test, $P > 0.05$) (Fig. 1D). The membrane hyperpolarization induced by the α_2-adrenoceptor agonist UK14304 (3 μM) was also the same in transgenic and wild-type mice (FLAGMOR-Tg/+ = 23.9 ± 1.6 mV, n = 14; wild type = 25.8 ± 0.9 mV, n = 24; t test, $P > 0.05$), suggesting that the higher receptor numbers in FLAGMOR-Tg/+ neurons did not interfere with activity of GIRK. Increased receptor expression in FLAGMOR-Tg/+ mice did, however, result in an increase in the potency of ME. There was a 7-fold leftward shift in the EC_{50} value in recordings made from slices taken from FLAGMOR-Tg/+ mice compared with wild type (FLAGMOR-Tg/+, EC_{50} = 94 nM; wild type, EC_{50} = 658 nM (Fig. 1D).

Hemizygous FLAGMOR-Tg/+ mice were crossed with MOR knockout (MOR−/−) mice for two purposes. First, if opioids caused a hyperpolarization in neurons in the compound mutant mice, this would demonstrate that FLAGMOR was functional in the absence of all endogenous MOR. Second, this genetic cross would decrease the total number of receptors on LC cells. The maximum hyperpolarization in neurons from compound mutant FLAGMOR-Tg/+, MOR−/− mice was similar (29.7 ± 1.4 mV; n = 15) to that observed in wild-type and FLAGMOR-Tg/+ mice. The EC_{50} value for ME in slices from the FLAGMOR-Tg/+, MOR−/− mice was 151 nM, 50% greater than that in the FLAGMOR-Tg/+ mice (94 nM) and only 4-fold less than the EC_{50} value of wild-type animals (658 nM) (Fig. 1D).

FLAGMOR Internalization in Brain Slices. A useful feature of the detection system combining a FLAG epitope-tagged receptor and M1 antibody is that the binding affinity between the two is decreased considerably upon the removal of calcium from the extracellular buffer. Slices were prepared identically to those used for electrophysiological experiments, incubated with the fluorescent M1 antibody for 45 min, and then placed in a tissue bath on the microscope for imaging. The fluorescent outlines of cell bodies were first visualized in each experiment (control in Fig. 2A). When the

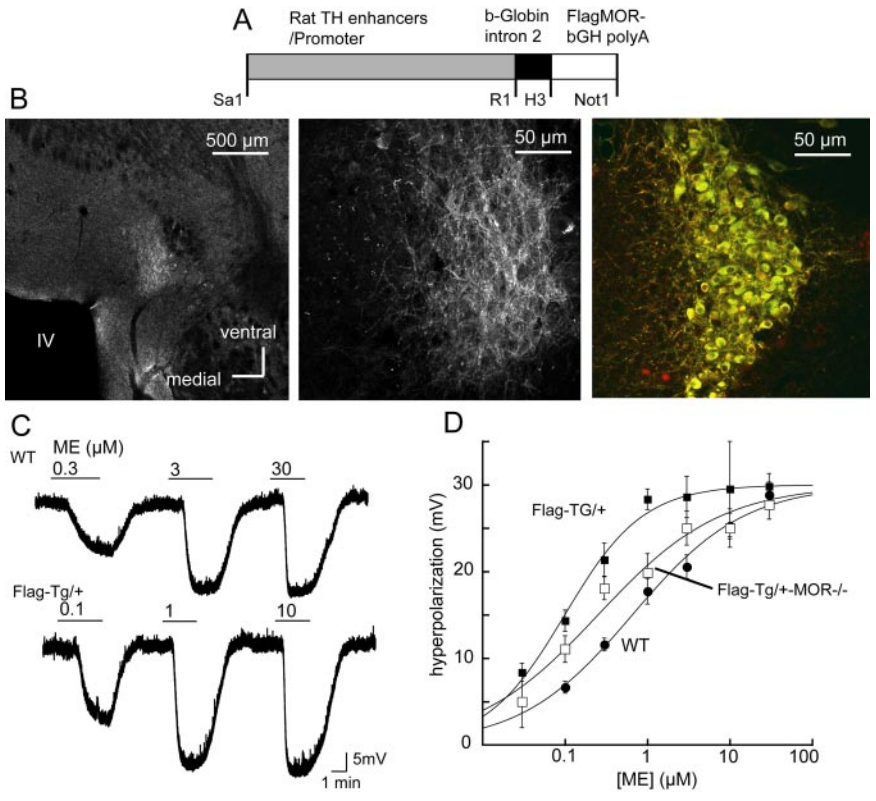

Fig. 1. Expression and signaling of FLAGMOR in LC neurons. A, restriction map of the TH-FLAGMOR-Tg construct. B, left, low-power micrograph of a slice containing the LC. The light area is the LC stained using the M1 antibody conjugated with Alexa 555. Middle, higher power image showing fluorescent staining of processes and cell bodies. Right, FLAGMOR was stained in red, and TH was stained with a secondary antibody labeled with Alexa 488. C, ME caused a concentration-dependent hyperpolarization in slices from wild-type and FLAGMOR-Tg/+ mice. Cells from the FLAGMOR-Tg/+ mice were more sensitive to ME. D, concentration-response curves for ME in slices from wild-type mice (●, EC_{50} = 653 nM), FLAGMOR-Tg/+ mice (■, EC_{50} = 94 nM), and compound mutant FLAGMOR-Tg/+, MOR−/− mice (□, EC_{50} = 151 nM).

superfusion solution was changed to a solution that was calcium-free (0.5 mM EGTA) for 10 min and the cells were imaged again, staining on the plasma membrane decreased to background levels (Fig. 2A). This result confirmed that the fluorescence seen surrounding the cell body required calcium and that a 10-min wash with calcium-free solution eliminated specific staining. In the next experiment, an initial image was taken following staining, and a second image was taken after treatment of the slice first with 30 μM ME for 15 min followed by calcium-free (EGTA) solution for 10 min. The resulting image showed numerous fluorescent puncta remaining within the soma and processes (Fig. 2, C and D). To determine whether the ME-induced increase in intracellular puncta was receptor-dependent, slices were treated with the irreversible opioid antagonist β-chlornaltrexamine (β-CNA) at 500 nM for 2 min before the application of 30 μM ME for 15 min. The slice was then treated with calcium-free (EGTA) solution, and the result was a complete block of receptor internalization (Fig. 2B; EGTA wash without ME treatment = 34.2 ± 2.9%, $n = 6$; EGTA wash after β-CNA + ME = 38.7 ± 4.3%, $n = 3$). The results indicate that the appearance of intracellular fluorescent puncta within the cell was dependent on the activation of FLAGMOR.

The concentration dependence of internalization induced by ME was determined in slices from FLAGMOR-Tg/+, MOR−/− mice (Fig. 3). Application of 300 nM ME did not result in an accumulation of fluorescence that was different from control. At higher concentrations (3 and 10 μM), more intracellular fluorescence was observed, and the concentration that was required to cause a half-maximal amount of internalization was about 3 μM, well above the EC_{50} value (151 nM) for the hyperpolarization. Thus, the concentrations of ME required to induce desensitization and internalization were significantly greater than that required to induce hyperpolarization (Harris and Williams, 1991), indicating that less receptor occupancy is required to mediate the activation of the potassium current.

The time course of accumulation of intracellular fluorescence was examined by capturing images at 3-min intervals following the onset of superfusion with 30 μM ME ($n = 4$) (Fig. 4). Within the first 3 min, there was a detectable decrease in fluorescence at the plasma membrane and an increase in cytoplasmic fluorescent. Cytoplasmic puncta continued to increase over 12 min. This result demonstrated that detectable FLAGMOR internalization was observed within 3 min, a period during which there is a significant amount of desensitization. With prolonged treatment, ME induced more cytoplasmic fluorescence in a perinuclear region.

Desensitization and Recovery from Desensitization. To determine whether FLAGMOR activity was regulated similarly to native receptors, desensitization was induced by a saturating concentration of ME (30 μM) for 10 min. Before the application of the desensitizing concentration of ME, a prepulse of ME (EC_{50} value) was applied, and the amplitude of the hyperpolarization was measured. Desensitization was measured in two ways. The first was to measure the decline in the peak hyperpolarization that occurred during the 10-min perfusion of 30 μM ME (Fig. 5A). The amplitude of the hyperpolarization after 10 min decreased to 70, 79, and 76% of the peak in wild-type, FLAGMOR-Tg/+, and FLAGMOR-Tg/+, MOR−/− mice, respectively (ANOVA, $P > 0.05$) (Fig. 5B). Second, the amplitude of the hyperpolarization induced by an EC_{50} concentration of ME tested 5 min after washout of the desensitizing concentration of ME was decreased to 38 ± 3% of the prepulse amplitude in wild-type mice (300 nM ME; $n = 9$ neurons), 35 ± 6% in FLAGMOR-Tg/+ mice (100 nM ME; $n = 5$), and 40 ± 7% in FLAGMOR-Tg/+, MOR−/− mice (100 nM ME; $n = 5$; ANOVA, $P > 0.05$).

The time course of recovery from desensitization was measured by repeated applications of ME (100 or 300 nM) over a 30-min period (Fig. 5, A and C). All three genotypes showed a similar extent and time course of recovery from desensitization (ANOVA, $P > 0.05$). Thus, FLAGMORs were functional and regulated similarly to endogenous receptors.

Recycling of receptors was observed in experiments where the preparation was treated with 30 μM ME for 5 min, followed by a wash with calcium-free EGTA buffer for 10 min and a 45-min wash with ACSF. The results show that fluorescent antibody-receptor complexes reappeared along the plasma membrane (Fig. 5D). Thus, the FLAGMOR/fluorescent M1-bound complex was capable of recycling in a time frame similar to the recovery from desensitization. Although only a fraction of the internalized receptors were able to traffic back to plasma membrane, the hyperpolarization induced by ME recovered almost completely. It is likely that the percentage of receptors that return to the plasma membrane is sufficient to result in a maximal hyperpolarization. The receptor-reserve reported in LC neurons suggests that only a small percentage of total receptors are necessary to elicit a maximal response (Christie et al., 1987).

Agonist Dependence of MOR Desensitization and Internalization. A series of opioid agonists were examined for

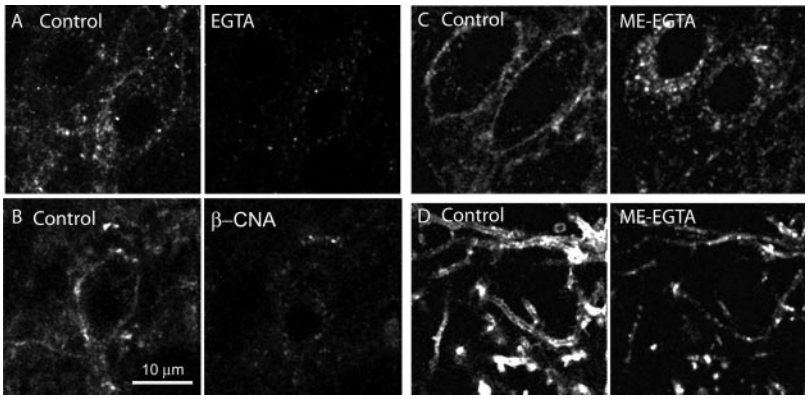

Fig. 2. Internalization of FLAGMORs induced by ME is receptor-dependent. A, control (left) shows the initial staining pattern with an Alexa 594-conjugated anti-FLAG antibody. Right, same slice after treatment with calcium-free (EGTA) solution. C and D, internalization of FLAGMOR induced by ME. Left, initial staining illustrating cell bodies (C) and dendrites (D). Right, staining following treatment of the slice with 30 μM ME for 15 min followed by calcium-free (EGTA) solution for 10 min. B, pretreatment with 500 nM β-CNA for 2 min blocked internalization induced by ME. Left, control and right, after 30 μM ME for 15 min. Scale bar, 10 μm.

the ability to induce desensitization and internalization in LC slices (Fig. 6). As has been reported in other systems, ME, methadone, and etorphine were all effective receptor-internalizing agents. The percentage of internalization induced by 30 μM ME was 35 ± 8% (n = 6), by 1 μM etorphine was 44 ± 9% (n = 6), and by 15 μM methadone was 30 ± 6% (n = 6). The internalization induced by morphine (6 ± 3%; n = 6), oxymorphone (5 ± 5%; n = 6), and oxycodone (2 ± 4%; n = 6) was not different from background.

The ability of each opioid agonist to induce hyperpolarization and acute desensitization was also compared (Fig. 6, B and C). Each of the agonists produced a similar maximal hyperpolarization. Desensitization was measured as the decline in the amplitude of the hyperpolarization induced by each agonist during a 15-min application of a saturating concentration. The washout of all agonists except ME was very slow, so reversal of the hyperpolarization was obtained with the perfusion of 1 μM naloxone. Following return of the membrane potential to control, the α-2-adrenoceptor agonist UK14304 at 3 μM was perfused, and the amplitude of the resulting hyperpolarization was measured. During a 15-min application of 30 μM ME, 1 μM etorphine, 15 μM morphine, and 15 μM oxymorphone, the hyperpolarization declined by 22 ± 4, 23 ± 3, 16 ± 1, and 24 ± 2%, respectively (Fig. 6C). Methadone (15 μM) caused the greatest decline in hyperpolarization (34 ± 8%); however, this was probably the result of a blockade of the potassium channel because the hyperpolarization induced by the α-2-adrenoceptor agonist UK14304 was also reduced (Rodriguez-Martin et al., 2008). The amplitude of the hyperpolarization induced by 3 μM UK14304 was 29.1 ± 1.0 (n = 20) following desensitization with all opioid agonists except methadone (17.8 ± 1.5; n = 5; P = 0.002). Thus, with the exception of methadone, the opioid-induced desensitization was homologous. Oxycodone (15 μM) resulted in a decline of only 9 ± 2%. The rank order for acute homologous desensitization was ME = etorphine = oxymorphone ≥ morphine ≫ oxycodone. Thus, oxymorphone and morphine caused desensitization without inducing internalization.

Internalization in β-Arrestin2-KO Mice. The elimination of β-arrestin might be expected to eliminate or at least decrease the amount of desensitization and internalization. Hemizygous FLAGMOR-Tg/+ mice were crossed with β-arrestin2-KO (Arr−/−) mice to generate compound mutant FLAGMOR-Tg/+, Arr−/− animals to determine the role of β-arrestin2 in desensitization and internalization. Intracellular recording of neurons in slices from Arr−/− animals showed that 30 μM ME induced a hyperpolarization to 26.7 ± 1.0 mV that declined to 76 ± 3% of its initial amplitude after 10-min application (Fig. 7B), values not significantly different from those observed in wild-type mice (t test, $P > 0.5$). Morphine-induced desensitization in slices from the Arr−/− animals and was not different from that in slices from wild-type animals (Arr−/− decreased to 84.6 ± 2.6% of the peak; wild type, 84.0 ± 1.0), despite the fact that the maximum hyperpolarization was reduced (13.5 ± 0.9 mV). Internalization induced by ME was examined in slices from FLAGMOR-Tg/+, Arr−/− animals. Slices from these mice were treated with 30 μM ME for 15 min, and the percentage of internalization was calculated. Internalization of FLAGMOR did not differ between littermates lacking or expressing β-arrestin2 (FLAGMOR-Tg/+, Arr−/− = 28 ± 3%; n = 9 neurons; FLAGMOR-Tg/+ = 26 ± 5%, n = 10) (Fig. 7, A and C). Thus, elimination of β-arrestin2 had no effect on desensitization or internalization.

Discussion

In the present study, brain slices from transgenic mice were used to examine desensitization and trafficking of μ-opioid receptors. Epitope-tagged (FLAG) μ-opioid receptors (FLAGMOR) were targeted to locus coeruleus neurons using regulatory elements from the tyrosine hydroxylase gene. Using ME, the FLAGMOR was activated and regulated in a similar way as the endogenous receptor. A series of opioid agonists were used to determine whether different agonists activated distinct pathways. All agonists activated a potassium conductance that resulted in the same maximum hyperpolarization. The demonstration of agonist-selective regulation of desensitization and internalization indicates that the two processes are independent and are not necessarily serial events. Originally, morphine was described as the agonist that caused neither desensitization nor internalization (Alvarez et al., 2002); however, recent results indicate that it can cause both desensitization (Borgland et al., 2003; Dang and Williams, 2005) and internalization (Haberstock-Debic et al., 2005). In contrast, the present results based on brain

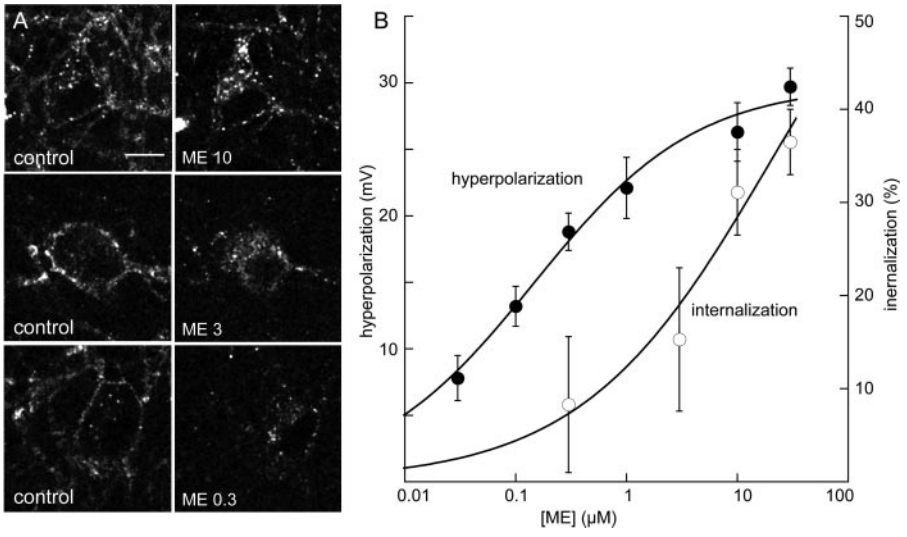

Fig. 3. Concentration dependence of internalization induced by ME. A, images were taken in control (left) and after treatment of slices with different concentrations of ME for 15 min followed by a wash with calcium-free solution (right). Scale bar, 10 μm. B, internalization required high concentrations of ME. The percentage of internalization caused by a 15-min application of ME is plotted and compared with the concentration response to the hyperpolarization. All experiments were carried out using slices from FLAGMOR-TG/+, MOR−/− animals.

slices containing LC neurons show that morphine causes desensitization but no internalization. Oxymorphone was similar to morphine in that it caused robust desensitization in LC neurons from rats (Virk and Williams, 2008) and mice but was ineffective at evoking internalization of FLAGMOR. The processes of internalization and desensitization have also been separated in experiments using cultured neurons treated with concanavalin A, where desensitization induced by ME and a dermorphin analog (dermorphin-Alexa 594) was induced in the absence of internalization (Arttamangkul et al., 2006). Thus, it seems that although desensitization may be a precursor to internalization, internalization is not necessary for the expression of desensitization.

The agonists used in this study produced three distinct classes of effects. One group caused both desensitization and internalization and included ME, etorphine, and methadone. Morphine and oxymorphone were distinct in that neither caused detectable internalization, but both were capable of inducing desensitization. Oxycodone was the only agonist that neither desensitized nor internalized the receptor. Although oxycodone and oxymorphone differ only in a single methyl group, there was a distinct functional difference between the two compounds. Oxymorphone was more effica-

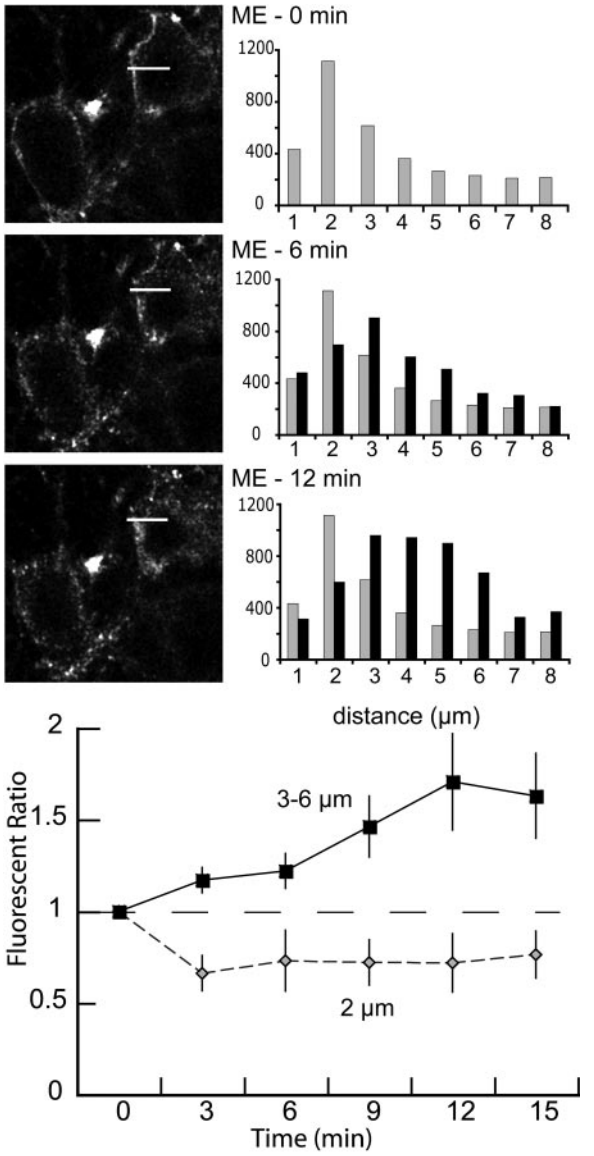

Fig. 4. The time course of receptor internalization. A, illustration of a single experiment showing the time course of internalization with images collected at 6-min intervals (top before ME, middle 6 min after ME, and bottom 12 min after ME). Each image is a single Z-scan (1 μm). The white bar in each image represented the area used to determine fluorescent intensity from the plot profile (0.5 $\times$ 8 μm; ImageJ). The fluorescence measured over a distance of 1 μm was added and plotted at the right for each image. Gray bars are the fluorescence measured before addition of ME. At 6 and 12 min, the fluorescence moved away from the plasma membrane into the interior of the cell. B, summary of the fluorescent ratio increasing in cytoplasm during 15-min perfusion of 30 μM ME (n = 4). The fluorescence ratio was determined by dividing the counts measured after the addition of ME by the counts in the same area before addition of ME. Fluorescence decreased at the plasma membrane (2 μm; gray diamonds) and increased in the interior of the cell (3–6 μm; solid squares).

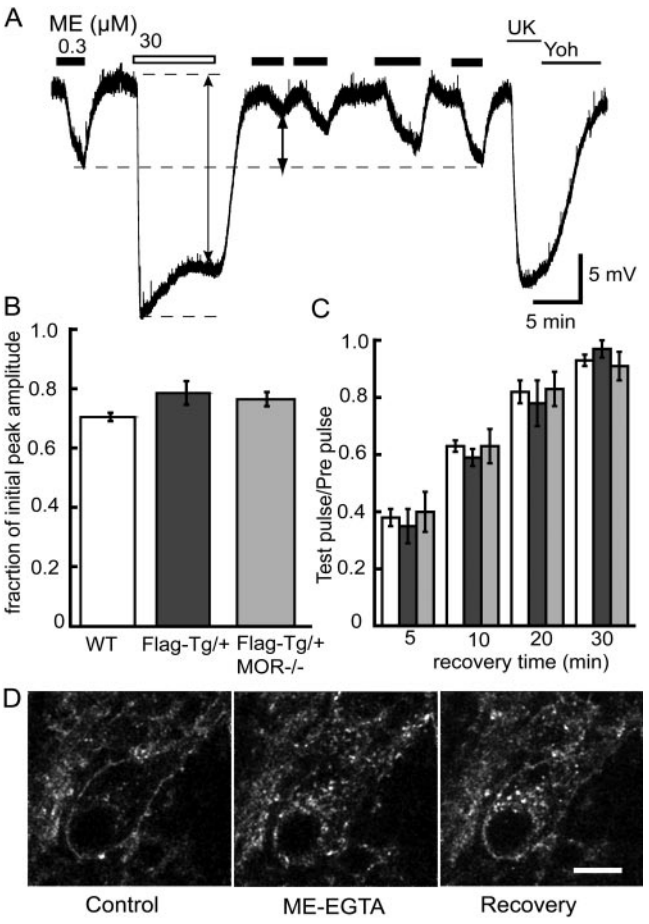

Fig. 5. Recovery from desensitization and reinsertion of FLAGMOR into plasma membrane. A, voltage trace from a wild-type mouse showing the hyperpolarization induced by 300 nM ME before and after treatment of the slice with 30 μM ME for 10 min. Following the washout of 30 μM ME, the hyperpolarization induced by 300 nM ME was depressed and recovered completely after 30 min. At the end of the recording, a saturating concentration of UK14304 (3 μM), an α2-adrenoceptor agonist, and its blockade by the antagonist yohimbine (Yoh), were tested. B, summary of the change in membrane potential during the 10-min treatment with 30 μM ME. The results indicate that the decline in the hyperpolarization was the same in slices from all three genotypes. C, summary of the time course and extent of recovery from desensitization. There was no difference between genotypes. Given that the FLAGMOR-Tg/+ and FLAGMOR-Tg/+ MOR$-/-$ were more sensitive to ME, the test concentration used to measure the recovery from desensitization was reduced to 100 nM. D, return of receptors to the plasma membrane of cell bodies. Left, initial staining. Middle, after application of 30 μM ME for 5 min and EGTA solution for 10 min. Right, after a 45-min wash. Scale bar, 10 μm.

cious and much more effective at inducing desensitization than oxycodone (Virk and Williams, 2008). Given that oxymorphone is a natural metabolite of oxycodone, the receptor-dependent processes of both oxycodone and oxymorphone must be considered in vivo.

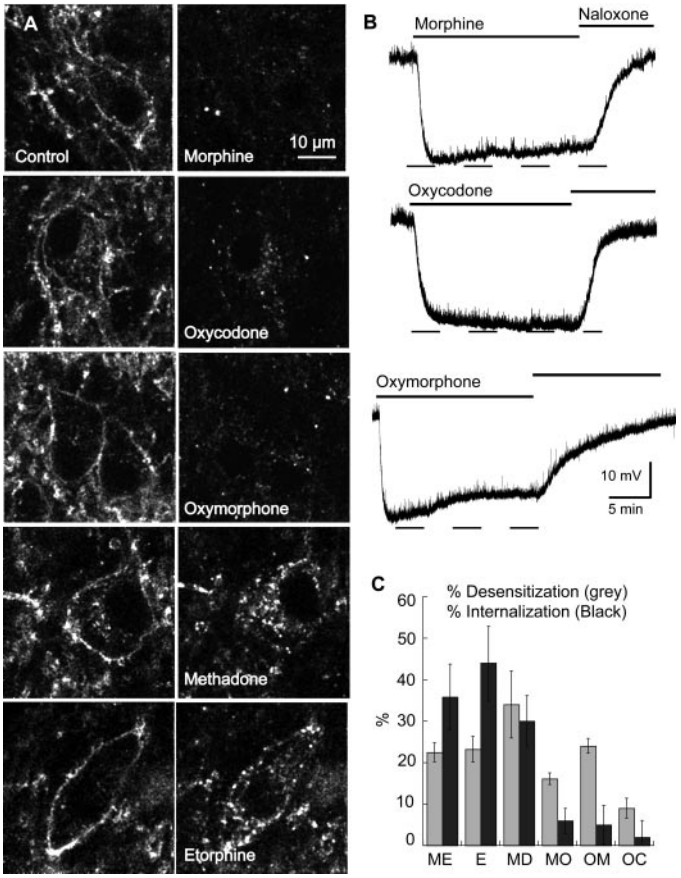

Fig. 6. Agonist-selective internalization. A, images of the internalization. Left, control; right, after agonist (15 min) followed by calcium-free (EGTA) wash. Etorphine (E) and methadone (MD) caused dramatic internalization, whereas morphine (MO), oxymorphone (OM), and oxycodone (OC) caused very little. B, example traces of the hyperpolarization induced by morphine, oxycodone, and oxymorphone (15 μM each), all agonists that caused little or no internalization. C, summary plot showing the decline in hyperpolarization (percentage of desensitization; gray bars) and internalization (percentage of internalization; black bars). The results show that different opioids produce different patterns of receptor regulation.

The only way that desensitization could be compared using a series of agonists was by measuring the decline in the hyperpolarization during an extended application of a saturating concentration of each agonist. The slow washout of most agonists from brain slices prevented a more quantitative measurement of a decrease in sensitivity that was possible with ME (Figs. 5A and 6B). The decline in peak hyperpolarization is not the most sensitive assay, because receptor reserve and the different efficacies of agonists result in a variable amount of desensitization. Given that near saturating concentrations of agonist are required for both desensitization and internalization, the decline in peak hyperpolarization would be blunted in experiments in which highly efficacious agonists, such as ME or etorphine, were examined. Thus, although a quantitative measure of desensitization is not possible, the rank order of the ability of various agonists to induce desensitization was reliably determined with this measure.

Morphine, oxymorphone, and oxycodone are small opiate alkaloids having morphinan base structure, whereas ME and methadone are linear and more flexible compounds. Considering the widely varying differences in chemical structure of opioid agonists, one may predict that different agonists could stabilize a range of receptor conformations and thus differentially affect the mechanisms leading to desensitization and internalization. Similar agonist-dependent effects on receptor trafficking of β2-adrenoceptors (Swaminath et al., 2005) and dopamine D1 receptors (Ryman-Rasmussen et al., 2007) have been observed. Likewise, distinctive signaling induced by opioid agonists can vary depending on, perhaps subtle, differences in experimental conditions. Morphine is a good example in that it does not cause μ-opioid receptor internalization in many cell types, but it did in cultured striatal neurons (Haberstock-Debic et al., 2005). It is also known that morphine can cause internalization in a system where G protein receptor kinase is overexpressed (Zhang et al., 1998). Although phospho-MOR preferentially interacts with and has higher affinity to β-arrestin2 than β-arrestin1 (Bohn et al., 2000, 2004; Oakley et al., 2000), the results with the β-arrestin2 KO mice suggest a possible interaction with β-ar-

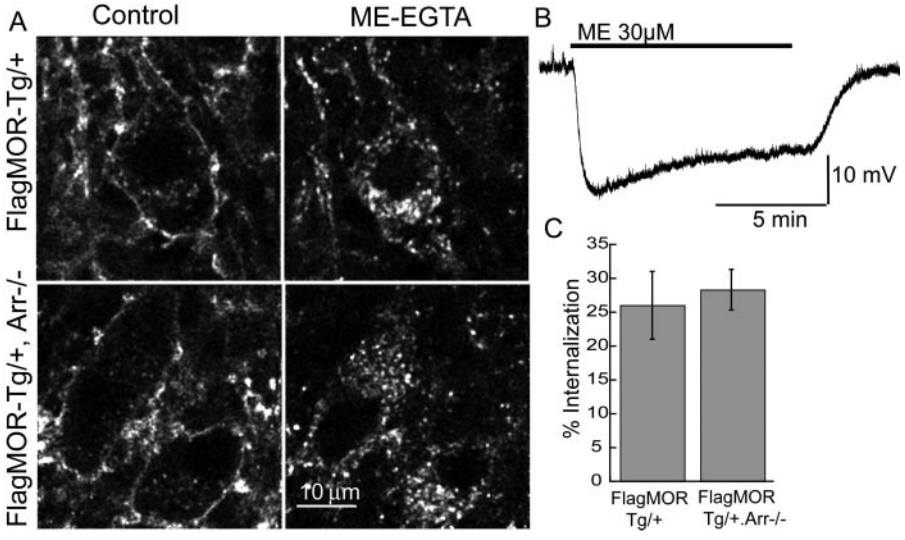

Fig. 7. ME-induced MOR internalization occurs in absence of β-arrestin2. A, image from FLAGMOR-Tg/+ (top) and FLAGMOR-Tg/+, Arr−/− LC slices in control (left) and following application of 30 μM ME for 15 min and calcium-free (EGTA) solution (right). Intracellular puncta were observed in both genotypes. B, intracellular recording of the hyperpolarization produced by application of 30 μM ME. The response declines to approximately 76% of the initial amplitude during the 10-min application. C, summary of the percentage of internalization caused by ME in FLAGMOR-Tg/+, Arr−/− and their FLAGMOR-Tg/+ littermates.

restin1. It is clear that mice lacking β-arrestin2 have a dramatically altered response to some opioids. For example, the tolerance to morphine is reduced, but treatment with etorphine resulted in tolerance that was the same as in wild-type animals (Bohn et al., 2004). These results are consistent with the idea that agonists can produce different patterns of receptor signaling in vivo (Stafford et al., 2001).

Although little work has been done at the cellular level with neurons from the β-arrestin2 knockout mice, the results in the present study are consistent with other studies that have reported that acute desensitization induced by opioid peptide agonists was not changed in either cultured dorsal root ganglion cells (Walwyn et al., 2007) or in LC neurons recorded in brain slices (V. E. Dang and M. J. Christie, personal communication). That desensitization and internalization of FLAGMOR were both observed in brain slices from β-arrestin2 knockout mice suggests that μ-opioid receptor signaling can occur by redundant pathways such that the control of receptor dependent signaling is ensured.

In summary, this study demonstrates the advantage of transgenic mice expressing an epitope-tagged receptor for the combined study of acute signaling through the activation of GIRK channels, desensitization, and receptor trafficking. This approach allowed direct study of three acute receptor-dependent processes in a brain slice preparation. The role that these early signaling pathways have in the development of tolerance and dependence remains the subject of controversy and intense interest. Given that all opioid agonists result in tolerance under a variety of conditions, it is clear that no one process can completely account for the whole animal response to opioids. It is also clear that a single opioid agonist, such as morphine, has variable actions that are dependent on the cell under study and even the part of the cell under study. With the understanding of the mechanisms that underlie the early events following receptor activation, it may be possible to develop a better understanding of the response of the whole animal to chronic opioid treatment.

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Address correspondence to: Dr. John T Williams, Vollum Institute, L474, Oregon Health Sciences University, 3181 W Sam Jackson Park Dr., Portland, OR 97239. E-mail: williamj@ohsu.edu